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We have examined the possible role of neu differentiation factor (NDF) in mammary tumorigenesis using transgenic mice. NDF is a ligand for ErbB4, a member of the ErbB family of tyrosine kinase receptors, of which two other members (EGFR and NEU) have been implicated in human breast cancer. Transgenic mice expressing a chimeric ligand having an NDFb-derived receptor binding domain within a TGFa backbone under an MMTV promote developed Harderian gland hyperplasias at high frequency. One mouse of 18 founders developed mammary carcinoma. Neither phenotype was transmissible to offspring. Other constructs, including NDFb2A, and mutant derived from that isoform, failed to express at significant levels in transgenic mice, precluding further analysis.

In a separate Task, we attempted to identify genes that cooperate with c-*neu* in mammary tumorigenesis by proviral tagging using MMTV that is passed from female C3H mice to offspring via their milk. However, the strain of C3H that we used has experienced phenotypic drift since its original derivation, and was found to have a very low incidence of mammary carcinoma for reasons that are not entrely clear. We did find evidence suggesting the presence of modifier loci in the FVB and/or C3H strains that influence the latency of *neu*-induced mammary tumors.

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NA In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

A S Palmer 3/18/98

PI - Signature Date

Annual Report for Grant Number DAMD17-94-J-4186
P.I.: Archibald S. Perkins, M.D., Ph.D.
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## TABLE OF CONTENTS

**Introduction**

Like most human cancer, breast cancer is the result of cumulative genetic alterations resulting in loss of growth control. The genes involved in this multistep process have not bee elucidated, but include p53, which is altered in more than 50% of cases, the Rb tumor suppressor gene, BRCA1 (5% of cases, mostly inherited, early onset), and the *neu/erbB-2/HER-2* gene in 20-30% of cases (Slamon et al. 1987). It is this last gene, *neu*, which is the focus of our research. *neu* is a member of a family of genes that encode receptor tyrosine kinases. Other family members include the epidermal growth factor receptor (*EGFR*), *erbB-3*, and *erbB-4*. Activated *neu* oncogenes are potent in transforming cells in culture and transgenic mice overexpressing either mutationally activated *or* normal *neu* in the mammary gland succumb to adenocarcinomas. The oncogenic effect of both activated and normal neu alleles was evident from whole animal studies (Muller et al. 1988). When normal c-*neu* gene was driven by MMTV in transgenic animals, the tumors were focal adenocarcinomas surrounded by hyperplasia, and were not pregnancy dependent (Guy et al. 1992). Since the mode of c-*neu* participation in oncogenesis in humans is amplification rather than activating mutations at c-*neu*, this transgenic model more closely resembles the situation in humans, and the long latency and stochastic nature of the tumors emphasizes the need for other events in carcinogenesis.

The $p185^{neu}$ receptor encoded by *neu* is stimulated by two families of ligands: the EGF family, and the NDF family, which includes heregulin (Holmes 1992), also known as neu differentiation factor (NDF) (Wen et al. 1992). None of the EGF family members appears to bind directly to $p185^{neu}$, yet several can activate the receptor via transmodulation. This is believed to occur by binding of the ligand to a high affinity receptor (e.g., EGFR) that then physically associates with $p185^{neu}$ and heterodimerizes. The result of this physical association is phosphorylation and activation of $p185^{neu}$ . Thus a variety of ligands can channel their signal through $p185^{neu}$, and the partners created depend on what other receptors are expressed in a given cell, and what ligands the cell is exposed to.

NDF is synthesized initially as a transmembrane glycoprotein with a 242 amino acid ectodomain that has an IgG-type motif and an EGF homology domain. The latter, contained in all members of the ErbB-binding ligand family, most likely functions in receptor binding. The

transmembrane form, via proteolysis at a site near the ecto-/transmembrane domain junction, is likely to be the precursor for the released form, as is the case for other membrane-bound growth factors.

While data is now emerging concerning the role of NDF in mammary carcinogenesis, considerably more is known concerning the role played other ligands that act through ErbB family members. Transgenic mice overexpressing TGFα, either with promoters targeting mammary epithelium, or generalized promoters, display mammary epithelial hyperplasia and neoplasia that is often malignant, and often involves the terminal ducts and secretory alveoli (Matsui et al. 1990). Recent studies show a potent interaction between TGFα and c-*neu* overexpression in transgenic mice: By crossing the MMTV-c-*neu* transgenics with MMTV-TGFα mice, a strong cooperativity was found, resulting in rapid hyperplasia and milk production (Muller, pers. comm). Clearly TGFα has a mitogenic, growth-stimulatory role in breast development and in mammary carcinogenesis. The role of NDFs is unclear, but given the finding that it can promote differentiation and growth cessation in cultured mammary epithelial cells, it may act antagonistically to TGFα. It is the goal of these studies to explore the role of NDFs in mammary carcinogenesis in whole animals using genetic approaches.

**Body**

Task 1. Overexpression of NDF: Transgenics. *neu* is one of the few genes clearly implicated in the development of human mammary tumors. In addition, TGFα, a ligand for a related growth factor receptor, EGFR, can stimulate $p185^{neu}$ activity via transmodulation, and can also play a stimulatory role in mammary carcinogenesis. We hypothesize that NDF, a putative ligand for $p185^{neu}$ that can stimulate its activity, but which induces the differentiation of mammary cell lines, plays an important role in mammary tumors, especially those in which *neu* also has a causative role. To address this hypothesis, we created transgenic mice with overexpression of NDF or NDF-derived chimeric constructs targeted to the mammary gland.

In collaboration with Dr. Neal Copeland of the NCI-Frederick, a series of transgenic mice were created to assess the oncogenic potential of NDF in the mammary gland. To this end, we made transgenic mice with NDF under transcriptional control of MMTV (14 founders) and under control of the whey acidic promoter (18 founders). For this experiment, we utilized the β2A isoform of NDF. As discussed above, this isoform has the β-type EGF homology domain that binds with higher affinity to the ErbB4 receptor (Wen et al. 1994), and the A isoforms contain the longest cytoplasmic domain. We chose this domain since it stimulates higher tyrosine kinase activity than the α isoforms, and because it was thought that the longer cytoplasmic tail may have some important role.

We have analyzed these mice for expression, and were disappointed to find that the level of stable transcripts in lactating or late pregnancy mammary glands was very low, especially when compared with that level obtained with the WAP promoter driving other genes, or the MMTV promoter driving TGFα (Matsui et al. 1990) (data not shown).. Nonetheless, we have treated a cohort of these mice with the mammary carcinogen dimethybenzanthracene (DMBA) in an effort to obtain a mammary tumor with this transgene for two reasons: one is to see if transgene expression increases in the tumor (as is often the case), and if so, to have mammary carcinoma cell lines that express NDF for future study. Seven transgenic mice were treated with DMBA and five of these developed mammary carcinomas. These were explanted into culture and frozen for further analysis, which will include RNA analysis for the

expression of the transgene, and karyotypic analysis by flourescence in situ hybridization (FISH).

We have also made several other types of transgenics. As discussed above, NDF is produced as a transmembrane protein that is later cleaved at a specific site on the ectodomain to release a soluble ligand molecule. This is similar to the situation with stem cell factor (SCF, also known as Steel factor or mast cell growth factor). With this factor, it has been shown that the membrane-bound and soluble forms have different activities, and are probably both essential in mouse development. We wondered if a form of NDF that could not be cleaved would have higher activity than one that can be cleaved. To test this, we created transgenic mice that contain MMTV-NDF with a point mutation in the proteolytic cleavage site that is used to generate the soluble form. Unfortunately, expression of the transgene could only be detected by reverse transcription-PCR, followed by Southern blotting. Nonetheless, the founders (no offspring) were maintained in mating, to determine susceptibility to mammary carcinomas. Out of fourteen mice aged to 18 months, none developed mammary carcinomas or other lesions of significance.

We were concerned about the problem of low expression of the transgene, and have approached this in several ways. One is that we obtained MMTV expression vectors from two other investigators (Phil Leder and Paul Jolicoeur), and constructed MMTV-NDF plasmids with these. We have transfected these into tissue culture cells and induced with dexamethasone (the MMTV LTR is responsive to dexamethasone). We compared the level of expression of these constructs to that seen with our transgenic construct, which was made with a vector from Robert Coffey. There was no significant difference in the level of expression between the different plasmids: all expressed at a fairly low level (data not shown).

These results are consistent with the notion that high level expression of NDF may be detrimental to the cell or the mouse, and is selected against. To test this, we have done the following. We know that the MMTV-TGFα plasmid from R. Coffey worked well in transgenics and in cell culture, so it is likely that this expression is not selected against. Since the receptor specificity of the ligand is determined by the EGF homology domain, we thought we could change the specificity of the MMTV-TGFα ligand by replacing its EGF homology domain with that from NDF (the β-type EGF homology domain). It was postulated that this chimeric protein, called

TNT (for TGFα-NDF-TGFα), would express better than NDF, yet have the same biologic activity of NDF. A converse swap was also made: replacing the EGF homology domain of NDF with that from TGFα (called NTN). We have used these to create transgenic mice, and have tested these for expression.

Of eighteen female MMTV-TNT founders, ten were examined for expression by Northern blot analysis of mammary tissue biopsies, using a radiolabeled probe for the transgene. Two were found to be positive: mouse 4330 and 4352 (data not shown). All eighteen were aged for tumors, and within three months six exhibited unilateral exophthalmos, which eventually became bilateral, resulting from progressive enlargement of the Harderian gland. This is a tubuloalveolar gland located within the orbit of many mammalian species though it is absent in primates. These were diagnosed histopathologically as hyperplastic adenomas. These lesions were noninvasive and nonmetastatic. RT-PCR analysis revealed that the transgene mRNA was present in the Harderian glands of affected animals but not in unaffected animals (data not shown). Unfortunately, in none of the dozens of offspring from these mice that were observed (for up to a year), did any Harderian gland adenomas develop. The reason for this nontransmission of phenotype is not clear.

It has been previously shown that MMTV-NDFβ2 expression leads to Harderian gland hyperplasia (Krane and Leder 1996). In the course of these studies, a report has been published describing the phenotype of transgenic mice having a MMTV-NDFβ2C transgene. (This NDF cDNA was cloned from a mouse mammary tumor, indicating that this isoform can be expressed in mammary tumors) (Krane and Leder 1996). The report describes a persistence of the terminal end buds in the mammary glands of virgin transgenic mice, and the presence of mammary gland adenocarcinomas in older mice.

While this report has diminished the novelty of our transgenic experiments, they suggest that NDF can play a role in mammary carcinomas in the mouse. Indeed, in one of the eighteen MMTV-TNT founders, (in which no transgene mRNA was detectable in the mammary gland) a mammary tumor developed with a latency of eight months. This tumor was transplantable in nude mice and could be grown in culture. Unfortunately, like the Harderian gland adenoma phenotype, this

susceptibility to mammary tumors was not transmissible in any of the offspring of this mouse (over ten observed), aged over a year.

The reciprocal construct, MMTV-NTN was also made and introduced into mouse zygotes for the derivation of transgenic mice. Sixteen transgenic female mice were identified, none of which were found to express the transgene. These mice were aged to 18 months, and none developed any noteworthy pathology.

Task 2. Targeted deletion of NDF via homologous recombination. In these experiments, we take another approach to testing the same hypothesis that NDF plays an important role in mammary development and neoplasia. If this hypothesis is true, then deletion of the gene encoding NDF should have effects on either mammary gland development or neoplasia, or both. It turns out that the creation of a null allele at the NDF locus is lethal in the homozygous state (Kramer et al. 1996; Meyer and Birchmeier 1996), and thus a null allele is uninformative in terms of the effect of NDF on mammary carcinoma. Thus, we considered creating more discrete mutations in the NDF gene via homologous recombination in embryonic stem cells, with the goal of creating "hypomorphic" alleles of NDF. The goal was to create mutations that are partially functional rather than null, and thus may give viability and an informative phenotype. However, due to our commitment to the other Tasks, we have not succeeded yet in completing the recombination constructs.

Task 3 Identification of protooncogenes that can cooperate with *neu*. It is clear from the studies of Muller and coworkers (Guy et al. 1992) that *neu* does not act alone in the generation of mammary carcinomas in transgenic mice. The long latency (5-8 months) and the solitary, stochastic nature of the tumors argues that other factors are necessary in the disease process. We thus hypothesized that while *neu* is an important oncogene in mammary tumorigenesis, other genes are involved. We proposed to identify what these other genetic factors are by retroviral mutagenesis and proviral tagging. This is was attempted by infection of transgenic MMTV-*cneu* mice with mouse mammary tumor virus (MMTV). We expected that infection of transgenic mice with the virus would cause an acceleration of tumorigenesis: a shortening of tumor latency, due to the activation of cellular genes that can cooperate with *cneu* in the development of tumors. The presence of the proviral tag in *cis* to the implicated oncogene would enable us to molecularly clone and characterize them.

To perform the experiment correctly, we backcrossed the MMTV-*cneu* transgene onto the C3H background for five generations, so that the genetic background will be essentially identical to C3H, the high mammary carcinoma strain that carries MMTV. After we had completed with this phase of the project, and had begun to age mice that had both the transgene and MMTV to look for acceleration of tumorigenesis, we received notification from the Jackson Labs that the strain of C3H that we had employed, namely the C3H/HeOuJ strain, no longer had the high incidence of mammary cancer that had been reported previously (Outzen et al. 1985) (see attached letter). Nonetheless, as we had already generated the cohorts of mice, we aged these for tumors.

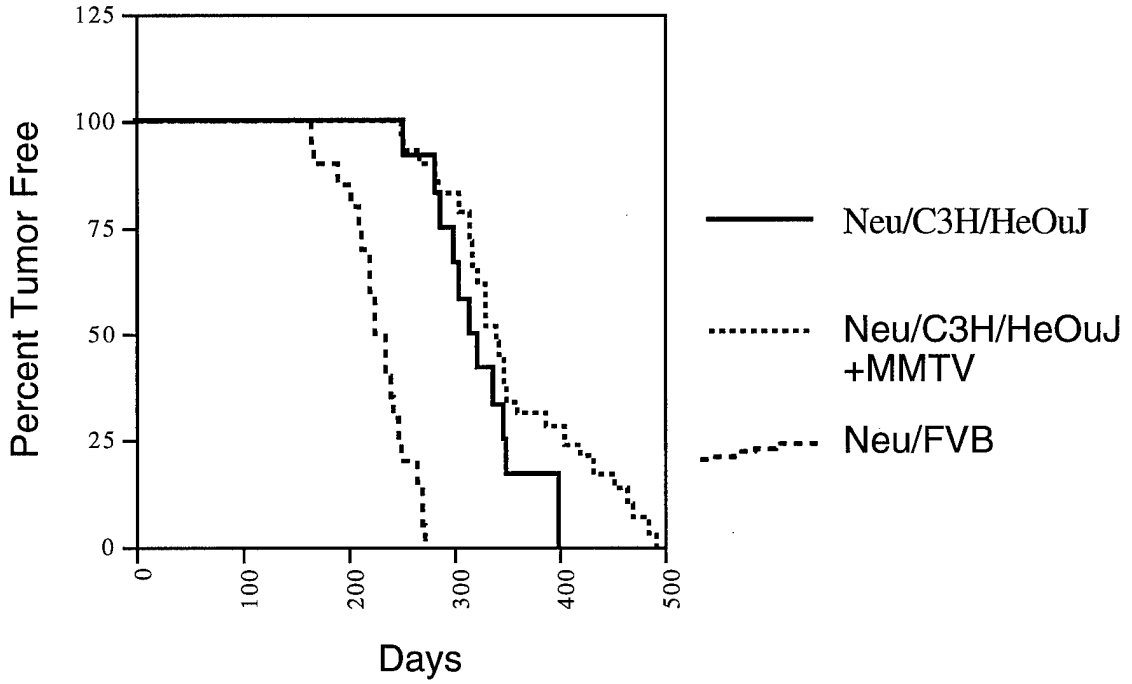
Four groups of mice were followed. Group 1 were offspring of C3H/HeOuJ mothers that had inherited the transgene (Neu+MMTV); Group 2 were transgene positive offspring that were MMTV negative (sired by C3H males; Neu alone); Group 3 were offspring of C3H females without the transgene (MMTV alone); Group 4 were negative for both virus and Neu transgene. Mice were kept pregnant and lactating.

Mice in the four groups were followed for up to 18 months, checking each week for the presence of mammary tumors by palpation. Time of tumor onset was noted, and mice were sacrificed and necropsied when moribund or at 18 months, whichever was sooner.

We were able to make two observations in this experiment. One is that we confirmed that the C3H/HeOuJ strain has a relatively low incidence of mammary cancers. Of the 23 mice that were followed, only six (26%) developed mammary tumors, with an average latency of 383 days. This compares with an incidence of nearly 100% and a median latency of 217 d reported by Outzen et al in 1985 (Outzen et al. 1985). The reason for this is not clear, although numerous possibilities have been investigated by investigators at the Jackson Labs. They have confirmed that there is virus present in milk from 100% of C3H/HeOuJ mice. They suspect that there may be attenuation of the milk-transmitted MMTV and a mutational change at the *Lps* locus. This change in phenotype has had a negative effect on our ability to carry out the Task as originally planned.

The second observation is that the latency of tumorigenesis due to the *Neu* transgene is dramatically delayed in the C3H/HeOuJ background. The

median latency for tumor onset on the FVB background was 230 days, while the same transgene on the C3H/HeOuJ background was 314 days when the mice were sired by a male C3H/HeOuJ (MMTV negative), and 338 days when sired by a C3H/HeOuJ female (MMTV positive).




This is an important and unexpected finding and is likely due to the presence of mammary tumor susceptibility genes in the FVB background or resistance genes in the C3H/HeOuJ background. To test this, we are performing additional crosses between the two strains to analyze the mode of inheritance the the delayed cancer phenotype. This may lead ultimately to the identification of genes that can modify the incidence of mammary carcinomas that are induced by a known oncogene, c-Neu. Such cancer modifier loci are of particular interest (e.g., (Drinkwater and Bennett 1991; MacPhee et al. 1995)) due to their importance in identifying groups of patients that have a higher or lower risk of cancer.

**Relationship to Statement of Work (SOW).**

Task 1. Overexpression of NDF: Transgenics.
- a. Construction of vectors: Done
- b. Microinjection/Analysis: Done
- c. Expansion of strain: Done
- d. Aging for Tumors: Done

Task 2. Knockout of *Ndf*:

a. Creation of construct: Not complete. Due to the publication of this knockout by two other groups, we decided to change the design of the experiment. Unfortunately, this revised design has proved much more challenging to execute than envisioned.

Task 3. Identification of protooncogenes that can cooperate with c-*neu*.
- a. Backcross to C3H: Done
- b. Aging of mice for tumors: Done

The need for steps c) and d) is obviated by the lack of tumor acceleration in the MMTV+ *neu* transgenic mice.

Please note that the SOW given above is what was proposed in the body of the Grant Application. However, in the SOW listed at the end of the Proposal (p24), there is another Task, specifically, to overexpress NDF via murine mammary fat pad implantation studies (listed as Task 2, which made the Task 2 of this report Task 3, and the Task 3 of this report Task 4).

Conclusions. In Task 1 we were able to confirm the results of Krane and Leder (Krane and Leder 1996) that expression of the beta isoform of NDF in transgenic mice under the MMTV promoter leads to increased susceptibility to Harderian gland adenomas and mammary carcinomas, although the latter phenotype was seen in only one of eighteen founders. We experienced two serious technical problems, however, that has precluded further progress on this Task. One is that the expression of the transgenes having the NDF backbone was very low, indicating that this moiety is likely selected against at some point in the mouse. Second, neither the Harderian gland adenoma phenotype nor the mammary carcinoma phenotype transmitted from the founders to the offspring to the next generation, despite transmission of the transgene. These technical hurdles have stymied our ability to examine the potential interaction between NDF and c-*neu* in mammary tumorigenesis.

In Task 3 we found that the C3H/HeOuJ strain had a much lower incidence and longer latency for mammary tumors than previously reported (Outzen et al. 1985). This has precluded our ability to identify cooperating oncogenes by proviral tagging mutagenesis as originally planned. We did find, however, a marked lengthening in the latency of c-*neu*-induced mammary tumors due to the C3H/HeOuJ background, indicating the presence of modifier loci in these strains that affect the susceptibility to mammary carcinomas caused by c-*neu*. This would be an interesting area for future investigation.

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*Laboratory Animal Health*

**To: Users of C3H/HeOuJ Mice** **Date: March 13, 1996**

**From: Rick Bedigian Director, Quality Control Testing Laboratories**

**Subj: Mammary Tumor Incidence in C3H/HeOuJ mice**

The Jackson Laboratory maintains four C3H substrains. Two of these, C3HeB/FeJ and C3H/HeSnJ, are free of milk-transmitted mammary tumor virus (MuMTV) and have relatively low mammary tumor incidence. The other two substrains, C3H/HeJ and C3H/HeOuJ, carry the MuMTV and develop mammary tumors.

A change in mammary tumor latency and incidence occurred in the C3H/HeJ strain (Outzen, H.C., et. al., JNCI 75:917, 1985). This reduction in tumor incidence in the C3H/HeJ strain is thought to be associated with the attenuation of the milk-transmitted MuMTV and to a mutational change at the *Lps* locus. The C3H/HeOuJ strain has been separated from the C3H/HeJ strain since 1952 and is characterized by a shorter latency and higher mammary tumor incidence than the C3H/HeJ strain.

In the past year we have received information from some investigators that the onset of tumor development is delayed and the frequency of tumors reduced in the C3H/HeOuJ strain. It is the intention of this update to notify investigators using C3H/HeOuJ mice for mammary tumor studies of this reported change in tumor incidence.

**What Are we Doing**

We have examined C3H/HeOuJ mice for the presence of MuMTV In all milk samples examined we detected the presence of virus by both immunologic and PCR tests. However, these tests will not discriminate between disease causing and attenuated virus. We are presently aging some C3H/HeOuJ retired breeders to determine the latency and tumor onset in our colony. Also, evidence has been presented that MuMTV utilize cells of the immune system in its infection pathway; exogenous MuMTV expression is associated with a deletion of the Vβ14+ T-cells (Tatyana, V.G., et.al., Cell 69:637, 1992; Ignatowicz, L., et.al., J Exp. Med. 175.917,1992). We have begun to analyze the level of Vβ14 T-cells in C3H substrains. To date we find that in some 8 week old C3H/HeOuJ mice there is a deletion of this T-cell subset whereas in others the value is comparable to controls. It is too soon to evaluate the significance of this data. It will be several months before we know if there is a correlation of T-cell deletion and tumor incidence in C3H/HeOuJ mice as has been reported for other high mammary tumor strains.



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We have not detected any variation in the Genetic profile of the four C3H substrains Our genetic screen includes twenty-three isoenzymes located over eleven chromosomes plus H2 haplotypes run against a panel of H2 allosera. To date we are not aware of any mutation that occurred in the C3H/HeOuJ strain that is associated with the reported drop in tumor incidence.

**What Can Be Done**

Some investigators have utilized the C3H/HeOuJ strain because of their interest in mammary tumorigenesis or cancer therapy. Unfortunately at this time we do not know the degree to which the mammary tumor incidence is altered nor do we know what factor(s) is contributing to the altered tumor incidence in C3H/HeOuJ mice. Results from the aging and immunological study hopefully will be informative and provide the insight needed to explain the altered tumor incidence. There are other mammary tumor models available through Animal Resources that might be a suitable substitute for C3H/HeOuJ mice. These mice are listed on The Jackson Laboratory Induced Mutant Resource Home Page (http://lena.jax.org/resources/ documents/imr/). If you are interested in exploring some of these models our Technical Support Staff is available to discuss these alternatives with you.

We are truly sorry for the inconvenience and disruption to your research program that this has caused. If you would like to discuss this situation further, contact Dr. Carol Linder, Technical Service Advisor at 1-800-422-MICE ext 6230 or by FAX 207-288-8982.